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Evaluation of the activities of toothpastes with different contents in the prevention of enamel demineralization: An *in vitro* study

Farklı içeriklerdeki diş macunlarının mine demineralizasyonunu önleme etkinliklerinin değerlendirilmesi: *İn vitro* çalışma

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ABSTRACT

Objective: The purpose of this study was to evaluate the efficacy of toothpastes in preventing initial caries.

Methods: Sixty extracted human molar teeth were used in the study. After the initial DIAGNOdent values of the teeth were recorded, the teeth were divided into 6 groups (n=10) and toothpastes containing arginine+fluoride, hydroxyapatite+fluoride, bioactive glass+fluoride, fluoride, and chitosan were applied. After the teeth were kept in demineralization solution for 6 hours, remineralization solution for 16 hours, and toothpaste mixture for 2 minutes every day for a total of 14 days, final DIAGNOdent measurements were made, and the teeth were sectioned in the buccolingual direction. Then, microhardness measurement was performed. Mineral loss results were obtained from these measurements. Moreover, scanning electron microscopy images were obtained from 1 sample of every group. The data obtained were evaluated statistically and at a significance level of $P < .05$.

Results: Maximum increase observed in the DIAGNOdent measurements was in the control group and minimum increase was in the hydroxyapatite group ($P=.000$). Maximum hardness values in all the depths were in the hydroxyapatite group, while the minimum increase was in the control group. When the mineral loss results were examined, remineralization occurred in the hydroxyapatite+fluoride, arginine+fluoride, bioactive glass+fluoride, and fluoride groups, respectively. Demineralization was detected in the chitosan and control groups. When the chitosan group was compared with the control group, however, demineralization was prevented ($P=.119$).

Conclusion: It is concluded that regular use of the toothpastes containing hydroxyapatite, bioactive glass, and arginine making synergic effect with fluoride may be useful in the prevention of the initial caries lesions.

Keywords: Demineralization, remineralization, toothpaste, DIAGNOdent Pen, cross-sectional microhardness

ÖZ

Amaç: Bu çalışmanın amacı; diş macunlarının başlangıç çürüklerini önleme etkinliklerini değerlendirmektir.

Yöntemler: Çalışmada 60 adet insan çekilmiş molar dişi kullanıldı. Dişlerin başlangıç DIAGNOdent değerleri ölçüldükten, sonra 6 gruba (n=10) ayrıldı ve arginin+florid, hidroksiapatit+florid, biyoaktif cam+florid, florid, kitosan içeren diş macunları uygulandı. Dişler 14 gün boyunca her gün 6 saat demineralizasyon solüsyonunda, 16 saat remineralizasyon solüsyonunda, 2 dakika da diş macunu karışımında bekletildikten sonra son DIAGNOdent ölçümleri yapılarak bukko lingual yönde kesildi ve mikrosertlik ölçümleri yapıldı. Mikrosertlik testi sonunda elde edilen değerlerden mineral kayıpları hesaplandı ve kantitatif olarak değerlendirildi. Ayrıca her gruptan birer örneğin

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de Taramalı Elektron Mikroskobu (SEM) görüntüleri elde edildi. Elde edilen veriler istatistiksel olarak ve anlamlılık $P < ,05$ düzeyinde değerlendirildi.

Bulgular: DIAGNOdent ölçümlerinde gözlenen en fazla artış kontrol uygulanılan grupta, en az artış ise hidroksiapatit grubunda gözlendi ($P = ,000$). Tüm derinliklerde en yüksek sertlik değerleri hidroksiapatit grubunda, en düşük artış ise kontrol grubundaydı. Mineral kaybı sonuçları incelendiğinde sırasıyla hidroksiapatit+florid, arjinin+florid, biyoaktif cam+florid, florid gurubunda remineralizasyon meydana geldiği belirlendi. Kitosan ve kontrol grubunda demineralizasyon olduğu saptandı. Kitosan grubu kontrol grubu ile kıyaslandığında ise demineralizasyonu engellediği sonucuna varıldı ($P = ,119$).

Sonuç: Başlangıç çürük lezyonlarının engellenmesinde florid ile sinerjik etki yapan hidroksiapatit, biyoaktif cam, arjinin içeren diş macunlarının düzenli olarak kullanılması faydalı olabileceği sonucuna varıldı.

Anahtar Kelimeler: Demineralizasyon, remineralizasyon, diş macunu, DIAGNOdent Pen, çapraz mikrosertlik

INTRODUCTION

The teeth are constantly undergoing demineralization and remineralization.[1] Featherstone[2] reported that the balance between demineralization and remineralization is directly proportional to the balance between pathological and protective factors. When pathological factors dominate, the balance may deteriorate toward demineralization. When demineralization progresses, initial caries lesions are formed, which is accepted as the first clinical sign of caries formation on the enamel surface. However, if before or during demineralization, protective factors dominate, demineralization stops, and remineralization begins.[2]

The initial enamel caries must be remineralized before demineralization proceeds and cavitation occurs; this is the basis of minimally invasive dentistry.[3] To prevent initial enamel caries, demineralization can be prevented, or remineralization can be enhanced.[4] One of the most important materials used for this purpose is fluoride. Frequent low doses of fluoride are more effective in terms of preventing decay than are high doses several times a year.[5] For this reason, fluoride has been added to the structure of toothpastes so that patients can apply it easily and frequently.[6] Other agents such as calcium sodium phosphosilicate (bioactive glass), calcium carbonate carriers (arginine bicarbonate), and nano-hydroxyapatite are used in combination with fluoride in toothpastes to precipitate salivary calcium and phosphate ions onto the tooth surface. Cheng et al[7] reported that arginine promoted enamel fluoride uptake when used in combination with fluoride, thereby contributing to resistance of enamel to carious demineralization. A recent study reported that toothpaste containing bioactive glass + fluoride significantly remineralized artificial caries lesions formed on the enamel surface better than toothpaste containing only fluoride.[8] Amaechi et al[9] reported that hydroxyapatite toothpaste was more effective than fluoridated toothpaste in preventing tooth demineralization. Another study reported that hydroxyapatite had a synergistic effect with fluoride.[10]

Fluoride ions have beneficial effects in the prevention of caries. However, overuse of fluoride toothpaste may increase the risk of fluorosis, especially in children and people living in particular geographic regions.[11] In such cases, it may be necessary to use non-fluoridated products to prevent demineralization and increase remineralization. A chitosan gel prepared by dissolving chitosan in acetic acid has been proposed as a preventive/therapeutic agent for caries.[12] It is also reported that chitosan acts as a barrier against acid penetration into tooth enamel, thus preventing demineralization.[13]

When the studies are examined, it has been reported that toothpastes containing fluoride, bioactive glass, arginine bicarbonate, nano-hydroxyapatite, and chitosan inhibit enamel demineralization.[7,13-15] However, there is no study in which all these toothpastes are used together, and their effectiveness is compared with each other. Therefore, the aim of this study is to evaluate and compare the in vitro efficacy of fluoride toothpastes containing hydroxyapatite, bioactive glass, and arginine and fluoride-free toothpastes containing chitosan to prevent the formation of initial enamel caries. The null hypothesis of this study is that toothpastes containing different remineralizing agents will not prevent initial dental caries.

MATERIALS AND METHODS

Ethics committee approval for this study was obtained from Gazi University Clinical Research Ethics Committee (research code no: 2016-29), and this study followed the recommendations of the Declaration of Helsinki and Decision.

Teeth Preparation and Storage

In this study, human third molars extracted in the last month for orthodontic reasons were used. Teeth cleared of debris were inspected under a stereoscopic microscope for visible cracks, white spot lesions, or hypoplasia and then stored in 0.1% thymol solution until the study started. A 3 × 3 mm window was left on the buccal enamel of the teeth, and all other surfaces were covered with 2 coats of nail polish (Colorama Maybelline, São Paulo, Brazil).

Toothpastes and Their Contents Used in the Study

Arginine + fluoride (Colgate Maximum Anti-rotten Sugar Acid Regulator—Colgate Palmolive Company), hydroxyapatite + fluoride (Signal Expert Protection Complete Care—Unilever), bioactive glass + fluoride (Sensodyne Repair and Protection—GlaxoSmithKline), only fluoride (Colgate Triple Action—Colgate Palmolive Company), and chitosan-containing toothpaste (Chitodent—Helmuth Focken Biotechnik, Sindelfingen, Germany) were used. All the fluoride-containing toothpastes had 1450 ppm sodium monofluorophosphate. The toothpastes used in the study and their contents are shown in Table 1.

The Initial Laser Fluorescence Measurements

The surface of the samples was examined using DIAGNOdent® (KaVo, Biberach, Germany). Samples with laser fluorescence measurements of 3-7 were selected, while those with >7 were discarded. The teeth were randomly divided into 6 groups (n = 10), and the initial laser fluorescence measurements were recorded. Measurements were repeated 3 times, and the highest value was recorded as the laser fluorescence score.

Table 1. The Toothpastes and Their Contents Used in This Study

Toothpaste/Manufacturer	Contents
A group/Colgate Maximum Anti-caries Sugar Acid Regulator—Colgate–Palmolive Company	Calcium carbonate, water, glycerin, sodium lauryl sulfate, **arginine, sodium mononofluorophosphate (1450 ppm F),** aroma, sodium bicarbonate, cellulose glue, tetrasodium pyrophosphate, benzyl alcohol, sodium saccharin, and sodium hydroxide
B group/Signal Expert Protection Complete Care—Unilever	Water, sorbitol, hydrate silica, potassium citrate, **hydroxyapatite,** PEG32, zinc citrate, sodium lauryl sulfate, **sodium mononofluorophosphate (1450 ppm F),** trisodium phosphate, cellulose glue, sodium hydroxide, sodium saccharin, and limonene
C group/Sensodyne Repair and Protection—GlaxoSmithKline	Glycerin, PEG8, silica, **calcium sodium phosphosilicate (Bioactive glass, NovaMin®), sodium mononofluorophosphate (1450 ppm F),** titanium dioxide, aroma, carbomer, sodium saccharin, limonene, sodium lauryl sulfate, and potassium acesulfame
D group/Colgate Triple Effect—Colgate Palmolive Company	**Sodyummonofluorofosfat (1450 ppm F),** calcium carbonate, water, sorbitol, hydrated silica, sodium laurylsulfate, aroma, cellulose glue, magnesium aluminum silicate, sodium carbonate, benzyl alcohol, sodium saccharin, sodium bicarbonate, eugenol, and limonene
E group/Chitodent—Helmuth Focken Biotechnik (Sindelfingen, Germany)	Water, sorbitol, hydrated silica, glycerin, **chitosan,** lactic acid, fennel, fruit oil, xanthan gum, potassium, glycerizate, and CI 77891
K group	Toothpaste application was not made on samples in the control group

Bold texts in contents indicate active ingredients of toothpastes. PEG8, propylene glycol cocoate.

Solution Preparation

The demineralization and remineralization solutions were described previously.[16] The demineralization solution (pH 4.4 adjusted using 1 M KOH) comprised 2.2 mM calcium chloride dihydrate ($CaCl_2$), 2.2 mM sodium hydrogen phosphate (NaH_2PO_4), and 50 mM acetic acid. The remineralization solution (pH 7.0 adjusted using 1 M KOH) comprised 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 150 mM KCl.

Demineralization–Remineralization Cycle

In this study, Featherstone's pH cycle model was used to simulate pH changes in the oral cavity.[17] The teeth were treated for 1 minute with toothpaste–water slurries (4.0 mL per tooth; 1 part toothpaste and 3 parts water, thoroughly mixed for 4 minutes). Then, the teeth were rinsed with deionized water and transferred to 40 mL of demineralizing solution per tooth for 6 hours at 37°C. After demineralization, teeth were rinsed with deionized water and then treated again with toothpaste–water slurries. Teeth were then rinsed with deionized water and transferred to 40 mL of remineralization solution per tooth for 16 hours at 37°C. Demineralization and remineralization solutions were prepared fresh daily. This pH cycle was repeated daily for 14 days (Figure 1). At the end of the experiment, the samples were stored in the remineralization solution.

Laser Fluorescence Measurements After pH Cycle

After the demineralization–remineralization cycle, the teeth were removed from the solutions, washed with distilled water, and dried, and laser fluorescence measurements were recorded.

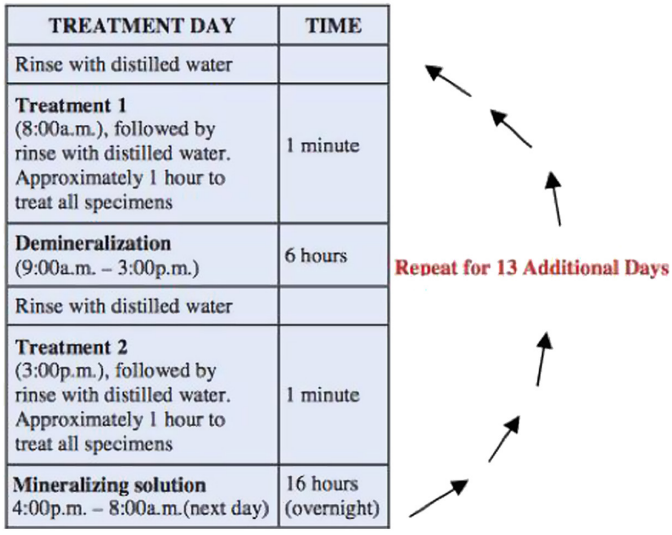

Figure 1. The application times of the pH cycle used in the study

Preparation of Teeth for Cross-sectional Microhardness Analysis

Teeth embedded in acrylic were divided into 2 in the buccolingual direction using a microtome tooth section (Figure 2). The surface of the sections was polished using 320-, 600-, and 1200 grit Al_2O_3 paper disks in water, washed with water, and dried. The samples were stored in capped cups containing wet cotton to prevent drying.

For cross-sectional microhardness analysis, microhardness from the external surface of the enamel toward the enamel–dentin junction was measured at intervals of 10 μm,[18] 20 μm,[19] and 25 μm[20,21] in previous studies.

In this study, microhardness was measured at 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, and 300 μm depths from the outer surface of the tooth to the inner parts, with a distance of 25 μm between the notches to prevent the notches from overlapping. The middle part of the window prepared on the tooth surface was marked to determine the point to start the measurement. Other measurement points were determined with the digitometer on the table. Microhardness was measured with a Vickers hardness device (Shimadzu, Kyoto, Japan) by applying 200 g of force for 5 seconds.

Mineral Loss Analysis

In this study, the formula reported by Featherstone et al[22] was used for mineral loss analysis. Mineral losses in each group were

Figure 2. Preparation of the tooth sample for cross-sectional microhardness measurement

analyzed from the microhardness values measured from all depths from 25 μm to 300 μm using the formula below:

$$\sqrt{VHN} = 0.197\ (\text{mineral volume percentage}) - 0.24$$

Delta Z = AUCtotal – AUC1

AUCtotal = 85 × (300 – 25) AUC1 = (25/3) × (V25 + 4 × (V50 + V100 + V150 + V200 + V250) + 2 × (V75 + V125 + V175 + V225) + V275) + (25/2) × (V275 + V300)

where VHN is the Vickers hardness value, *V* is the mineral volume percentage, delta *Z* is the mineral loss, and AUC (area under the curve) = The bioavailability of the toothpaste's active ingredient.

Scanning Electron Microscopy

One sample from each group was air dried, fixed on aluminum plates, and plated with gold. Then, surface topography images were taken with a field-emission scanning electron microscope (Hitachi SU5000, Hi-Tech., Ltd., Tokyo, Japan) at 2500× magnification with 10 kV acceleration voltage.

Statistical Analysis

Statistical Package for Social Sciences statistical software version 16 (IBM Corp., SPSS Inc.) was used for data analysis. Kolmogorov–Smirnov and Shapiro–Wilk tests showed that the data were normally distributed. One-way analysis of variance was used for between-group analysis, and the Tukey honestly significant difference test was used to identify the groups that were different. Correlations between the DIAGNOdent and mineral loss results were assessed by the Pearson correlation test. Significance was evaluated at the $P < .05$ level.

RESULTS

Laser Fluorescence Measurements

The first DIAGNOdent values did not differ significantly among the groups ($P > .05$). However, the values of each group before differed significantly from those after the pH cycle ($P = .000$). In all groups, the last DIAGNOdent values were significantly higher than the first values, and the differences were greatest and least in groups K and B, respectively.

The DIAGNOdent values of the control group differed significantly from those of the other groups ($P = .000$), but the values did not differ significantly among the toothpaste groups ($P > .05$) (Table 2).

Mineral Loss Results

The microhardness values at 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, and 300 μm depths are listed in Table 3.

Differences among the groups were significant at all depths ($P = .000$). Mineral losses were calculated from the hardness values obtained at all depths, and the increases and decreases in mineral contents differed significantly among the groups ($P = .000$). Mineral quantities increased in groups A, B, C, and D and decreased in groups K and E. The highest increase in mineral content was observed in group B, followed by groups A, C, and D, respectively, while a significant decrease in mineral amount was observed in group K (Table 4, Figure 3).

Correlation of Laser Fluorescence Measurements and Mineral Loss Results

There was a significant positive correlation between the DIAGNOdent values and the mineral loss results ($P = .000$) (Table 5).

Scanning Electron Microscopy Examination

In the control group, the enamel surface was porous and showed homogeneous demineralization. In the arginine, hydroxyapatite, bioactive glass, and fluoride groups, it was observed that the porosity seen in the enamel in the control group disappeared, and a bumpy film layer was formed on the tooth surface and remineralization was in the form of dense precipitation. It was observed that dissolution occurred in the center of the enamel prisms in the chitosan group, but there was no intense porosity as in the control group (Figure 4).

Table 2. DIAGNOdent Values Before and After the pH Cycle

	The first DIAGNOdent measurement (T0)		The last DIAGNOdent measurement (T1)		T1 – T0	
Groups	Mean ± SD	*P*	Mean ± SD	*P**	The Mean Difference ± SD	*P**
A	3.20 ± 0.45	.453	4.70 ± 0.48	.000	3.20 ± 0.45	.000
B	3.70 ± 0.48		4.90 ± 0.63		3.70 ± 0.48	
C	3.30 ± 0.68		5.20 ± 0.66		3.30 ± 0.68	
D	3.80 ± 0.45		5.90 ± 0.71		3.80 ± 0.45	
E	3.50 ± 0.61		6.00 ± 0.67		3.50 ± 0.61	
K	3.60 ± 0.50		11.30 ± 2.31		3.60 ± 0.50	

*Significant difference.

Table 4. Mineral Loss Results

Groups	Mean ± SD	*P*
A	−4280.75 ± 2408.14	.000***
B	−7367.72 ± 1288.25	
C	−2276.72 ± 1819.88	
D	−1214.44 ± 2355.97	
E	707.49 ± 2333.09	
K	4283.66 ± 2762.43	

***Significant difference. ***Extremely significant, **Very significant, *Significant.
***$P < .001$, **$P < .01$, *$P < .05$.

Table 3. Microhardness Values at Depths of 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, and 300 μm

Depth	Group A (Mean ± SD)	Group B (Mean ± SD)	Group C (Mean ± SD)	Group D (Mean ± SD)	Group E (Mean ± SD)	Group K (Mean ± SD)	*P*
25 μm	119.25 ± 32.73	197.60 ± 22.22	106.59 ± 17.31	94.20 ± 18.61	73.43 ± 17.24	57.22 ± 7.89	.000
50 μm	181.49 ± 81.97	310.60 ± 60.28	147.99 ± 50.11	139.01 ± 50.26	100.56 ± 42.92	63.72 ± 8.80	.000
75 μm	220.59 ± 94.64	382.00 ± 60.52	216.08 ± 74.77	210.04 ± 88.45	128.30 ± 69.75	71.86 ± 10.23	.000
100 μm	323.30 ± 116.51	459.20 ± 61.98	260.40 ± 80.81	242.19 ± 87.83	163.19 ± 92.02	120.93 ± 52.15	.000
125 μm	403.70 ± 98.34	485.4 ± 67.81	295.50 ± 82.09	285.27 ± 89.70	202.35 ± 97.02	136.41 ± 62.72	.000
150 μm	455.30 ± 69.46	514.50 ± 56.10	353.10 ± 79.50	334.9 ± 73.76	226.96 ± 83.22	168.13 ± 80.80	.000
175 μm	472.30 ± 60.77	529.60 ± 34.75	390 ± 58.88	367.20 ± 59.99	286.68 ± 84.27	200.16 ± 86.74	.000
200 μm	493.10 ± 49.13	538.80 ± 41.59	427.70 ± 40	390.90 ± 57.15	380.60 ± 45.25	258.50 ± 91.84	.000
225 μm	507.40 ± 50.61	556 ± 36.83	458.10 ± 30.20	409.90 ± 57.14	434.10 ± 43.05	290.9 ± 106.99	.000
250 μm	517.30 ± 45.55	561.20 ± 31.87	474.30 ± 37.47	432.30 ± 52.01	461 ± 42.44	343.90 ± 98.72	.000
275 μm	543.50 ± 50.54	570.20 ± 32.12	493.20 ± 30.56	447.30 ± 50.52	471.50 ± 36.67	383.80 ± 95.51	.000
300 μm	568 ± 51.97	586.20 ± 28.30	505.40 ± 29.32	462.60 ± 42.87	493.50 ± 32.99	407.60 ± 103.4	.000

Differences among the groups were significant at all depths ($P = .000$).

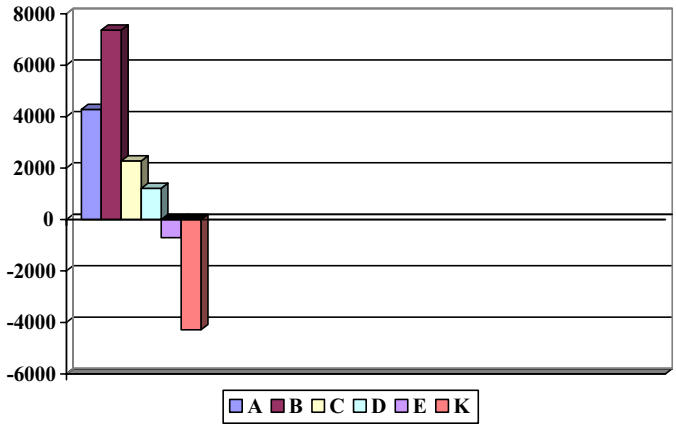

Figure 3. The comparison of mineral loss results between groups

Table 5. Correlations Between DIAGNOdent Values and Mineral Loss Results

	Mineral Loss Results	
Parameters	***r***	***P* (significance)**
DIAGNOdent results	0.666	DIAGNOdent results

R, correlation coefficient. ***$P < .001$, **$P < .01$, *$P < .05$
***Extremely significant, **Very significant, *Significant.

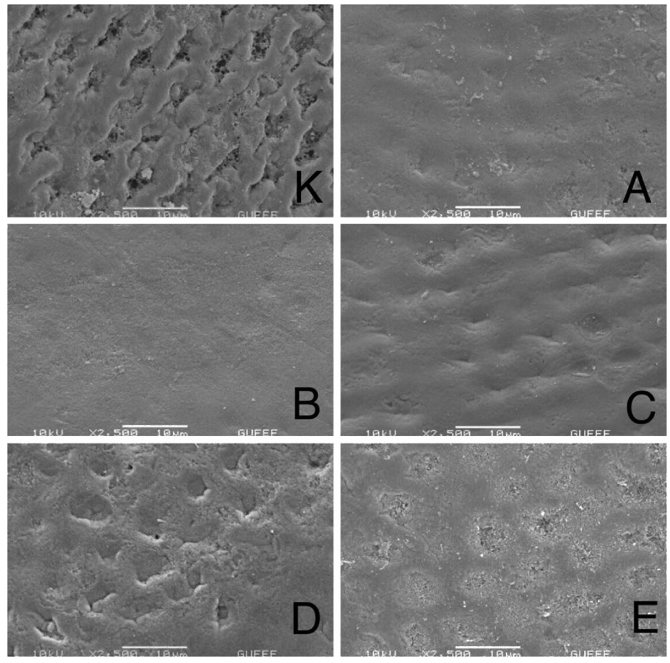

Figure 4. Scanning electron microscopy images of the groups (2500×)

DISCUSSION

Dental caries is still one of the most common chronic diseases today.[23] Considering both the evidence of the relationship between oral diseases and many general health problems and the effects of dental caries on the health system and the country's economy, there is a need for more research on caries prevention methods.

Toothbrushing and flossing are the gold standards in plaque control.[24] In addition, toothbrushing the teeth with fluoride toothpaste helps prevent cavities.[25] Axelsson et al[26] reported that dental caries can be prevented by regular toothbrushing and flossing. When the studies are examined, it has been reported that toothpastes containing fluoride, bioactive glass, arginine bicarbonate, nano-hydroxyapatite, and chitosan inhibit enamel demineralization.[7,14,27,28] However, there is no study in which all these toothpastes are used together, and their effectiveness is compared with each other. The aim of this study is to evaluate the caries prevention effectiveness of toothpastes with various contents, which are the most easily accessible individual oral and dental health care products of the society, and to compare them with each other.

In in vivo studies, determining the cause of treatment failure and controlling for individual variables such as dietary habits, oral-care habits, fluoride sources, and saliva volume and composition is difficult. In vitro studies facilitate the acquisition of numerical data for designing clinical trials[29] and are more rapid and less costly than in vivo studies.[30]

The teeth are constantly exposed to demineralization and remineralization due to changes in the oral cavity pH.[1] The in vitro pH cycle model simulates the dynamic conditions and natural processes in the mouth and can be used to evaluate the effects of materials on caries demineralization/remineralization.[16,29] Artificially formed caries-like lesions have all important histological features of natural caries and are thus used in in vitro studies of enamel demineralization–remineralization.[31]

Toothpastes can contain 4 types of fluoride compounds: sodium fluorophosphate, sodium fluoride, stannous fluoride, and amine fluoride. The different solubilities of these fluoride compounds result in the generation of different amounts of calcium fluoride, which affects enamel demineralization–remineralization and fluoride bioavailability.[32] Toothpastes with different fluoride compounds affect remineralization differently.[33] Thus, all fluoride-containing toothpastes used in this study had 1450 ppm sodium monofluorophosphate.

In restorative dentistry, in vivo and in vitro methods can be used to determine mineral loss and/or the depth of caries lesions such as quantitative light-induced fluorescence, laser fluorescence, optic caries monitoring, digital imaging with fiber-optic transfillumination, polarized light microscopy, profilometry, transverse microradiography, electrical transmission testing, microhardness testing, and scanning electron microscopy. Laser fluorescence devices (DIAGNOdent and DIAGNOdent Pen) are suitable for determining the demineralization and remineralization process of initial caries.[34] Comparing the in vivo and in vitro performances of DIAGNOdent, its accuracy in in vitro conditions is higher.[35]

Enamel hardness is related to its mineral content, so microhardness testing using the Knoop[7] or Vickers[36] hardness device is performed to assess enamel demineralization–remineralization.[22] In a study, the correlation between microhardness and volumepercentage of mineral content was 0.91.[37] For these reasons, DIGNOdent Pen and Vickers microhardness test were used as a quantitative technique to quantify enamel demineralization in this study.

The DIAGNOdent Pen values in all groups increased significantly after the pH cycle ($P = .000$). Also, the microhardness values increased with increasing depth up to 300 μm, possibly due to the decrease in the destructive effect of demineralization as the depth increases. The microhardness values of all toothpaste groups at all depths were higher than the control group. The DIAGNOdent and mineral loss results were significantly positively

correlated ($P = .000$). These results show that toothpastes with various contents are effective in preventing demineralization, and thus the null hypothesis of the study was rejected.

The arginine + fluoride-containing toothpaste was more effective in preventing demineralization than was the control toothpaste, as reported by Kraivaphan et al.[38] Moreover, the DIAGNOdent and mineral loss results differed significantly between the arginine + fluoride group and control groups but not between the fluoride-only group. It is reported that a toothpaste containing 1.5% arginine and 1450 ppm fluoride has greater remineralization activity than a toothpaste containing only 1450 ppm fluoride.[39] Ammonia produced as a result of metabolizing arginine by arginolytic bacteria neutralizes acids produced by pathological bacteria and increases plaque pH. In addition, the insoluble calcium contained in the toothpaste increases remineralization as it acts as a free calcium source. This explains why no such difference was detected in this study, although arginine + fluoride-containing and fluoride-only toothpastes showed significantly different demineralization prevention effectiveness in clinical trials.

The DIAGNOdent and mineral loss results differed significantly among the hydroxyapatite + fluoride, control and fluoride groups ($P < .05$). Toothpaste containing hydroxyapatite + fluoride prevented demineralization and provided the highest remineralization compared to other toothpastes in this study consistent with the results of previous studies.[40-42] Therefore, toothpastes containing hydroxyapatite + fluoride can be used to simultaneously prevent and treat caries. Application of nano-hydroxyapatite crystals for 10 seconds results in their penetration to deeper parts of demineralized subsurface lesions and the formation of structures that act as reservoirs for calcium and phosphate ions. This structure makes ions available during the caries process, thus maintaining mineral oversaturation of the enamel[41]; this may explain the efficacy of the hydroxyapatite toothpaste used in this study. It is also reported that complete remineralization of the lesion is not possible because the highly mineralized surface layer prevents the minerals from fully reaching the deeper parts of the lesion.[41,43] In this study, the lowest increase in laser fluorescence values in the hydroxyapaptide + fluoride group compared to the other groups supports the conclusion that hydroxyapatite does not fully provide remineralization.

The bioactive glass + fluoride and control groups showed significantly different DIAGNOdent, cross-sectional microhardness, and mineral loss results ($P < .05$) but not compared to the fluoride-only group. Yli-Urpo et al[44] reported that bioactive glass had a significant antimicrobial effect on Mutans Streptococcus and increased the pH of the oral cavity. This study's in vitro nature may explain that the bioactive glass + fluoride toothpaste exhibited greater remineralization activity than the fluoride-only toothpaste, but the difference was not significant. According to the results of this study, bioactive glass-based toothpaste may prevent the development of caries lesions, confirming previous reports.[14,32,45]

The mineral loss results revealed that chitosan-containing toothpaste did not provide remineralization compared to other toothpastes, but it did reduce demineralization compared to the control group. This result is consistent with the report of Uysal et al[36] that chitosan-containing toothpastes show greater inhibition of enamel demineralization than non-fluoride toothpastes. One of the limitations of this study is that the chitosan toothpaste does not contain fluoride, although the other toothpastes in the study contain 1450 ppm fluoride. The chitosan-containing toothpaste had the lowest demineralization prevention activity, possibly because it does not contain fluoride. Also, chitosan interacts with bacterial cell walls and membranes and exhibits bactericidal activity against *S. mutans*, the agent of dental caries.[46] This study's in vitro nature may explain that chitosan-containing toothpaste may have shown lower activity than other toothpastes. However, chitosan can bind to the salivary pellicles and form a multilayered layer in the presence of mucin.[47] These properties and its antibacterial activity on *S. mutans* suggest that chitosan-containing toothpaste will exhibit greater efficacy in vivo.

Because both of bioactive glass and chitosan on Mutans Streptococcus, the causative agents of caries, and the ability of ammonia produced by the metabolization of arginine by bacteria to increase plaque pH cannot be evaluated in vitro studies and cause different results. Therefore, further in vivo and in vitro studies are needed on this subject.

Within the limits of this study, it was determined that toothpastes containing hydroxyapatite, arginine, bioactive glass, fluoride, and chitosan were effective in preventing enamel demineralization.

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